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Characterization of two *Bacillus thuringiensis* plasmids whose replication is thermosensitive in *B. subtilis*

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1. SUMMARY

Two cryptic plasmids of 8.6 and 15 kb, originating from *Bacillus thuringiensis*, have been cloned in *Escherichia coli*. The determination of their physical map shows that the 8.6-kb plasmid harbors the transposon Tn*4430* and that the 15-kb plasmid carries Tn*4430* plus one copy of the IS*231* element. The replication regions were identified on the restriction maps and the segregational stability of derived plasmids containing these regions was analyzed in *B. subtilis*. The results indicate that the stability of these plasmids is negatively correlated to the temperature. After 30 generations, without selective pressure at 51°C, the two types of plasmids are lost.

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2. INTRODUCTION

During recent years, studies on the gram-positive entomopathogenic bacterium *Bacillus thuringiensis* have revealed the presence of complex arrays of resident plasmids in most of the strains examined [1–3]. Molecular hybridization experiments showed that these plasmids fell into two size groups in which there is partial conservation of DNA sequences [2]. Among the plasmids larger than 20 kb, some harbor insecticidal δ-endotoxin genes [3], whereas no function with the plasmids inferior to 20 kb has been shown. The striking conservation of cryptic plasmids in the species *B. thuringiensis* implies the presence of very efficacious replication functions for their stable maintenance.

In contrast to this stability of the plasmids in natural conditions or when bacterial cultures are developed at 30°C, growth at 42°C may be used to generate cured derivatives in which plasmids are lost [4]. This suggests a thermosensitivity of the functions of replication. These characteristics, the stability and the thermosensitivity of plasmid

vectors are important features both in biotechnology to introduce recombinant δ-endotoxin genes in *B. thuringiensis* and for genetic studies of gram-positive bacteria. Moreover, several of these plasmids have been shown to harbor different transposable elements [5,6] which can constitute convenient tools for insertional mutagenesis experiments.

In this paper, we report the isolation of two resident plasmids of *B. thuringiensis*, one of which carries the transposon Tn*4430* previously described [7] and the other bears Tn*4430* and an insertion sequence, IS*231*a, belonging to the IS*231* family [8,9]. The segregational stability of the two plasmids is analyzed in *B. subtilis* strain 168 at different temperatures.

3. MATERIALS AND METHODS

3.1. Bacterial strains and plasmids

The *B. thuringiensis* strain *thuringiensis* LM2 was isolated in our laboratory from an infected coleopteran larva and is referenced as serotype 1 by the WHO Collaborating Center for Entomopathogenic Bacillus held by H. de Barjac at the Pasteur Institute.

The *B. subtilis* strain 168 (*trpC*2) [10] and the *Escherichia coli* strain HB101 ($r_k^- m_k^-$ *recA*$^-$ *str*) [11] were used in this work.

The source and relevant characteristics of the plasmids used in this study are listed in Table 1.

3.2. Enzymes

T4 DNA ligase, DNA polymerase 1 large fragment and restriction enzymes (except *Asp*718) were from New England Biolabs (Beverly, MA, U.S.A.). *Asp*718 endonuclease was from Boehringer Mannheim (F.R.G.). Digestion of DNA was carried out in TA buffer (33 mM Tris–acetic acid, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol), except for *Bal*I enzyme which is used according to the manufacturer's instructions.

3.3. Transformation procedures

Transformation of *B. subtilis* and *E. coli* strains was carried out as previously described [10,12].

Table 1

Plasmids used

Plasmids	Size (kb)	Phenotype [a]	Replication		Construction/origin
			E. c.	B. s.	
pJH101	5.4	Ap Tc Cm	+	−	pBR322 + 1-kb fragment containing the chloramphenicol acetyl transferase gene of pC194 [13]
pHV33	7.3	Ap Tc Cm	+	+	pBR322 + pC194 [14]
pHT1001	14	Ap Cm	+	+	pJH101 + pHT1000; cloning at the *Bam*HI site
pHT1002	14	Tc Cm	+	+	pJH101 + pHT1000; cloning at the *Pst*I site
pHT1004	9.8	Ap Cm	+	+	pHT1001 deleted of the 4.2-kb *Kpn*I fragment containing Tn*4430*
pHT1005	7.5	Ap Cm	+	+	The 2.1-kb *Kpn*I-*Bam*HI fragment containing the replication region of pHT1000 is inserted between the *Eco*RV and *Bam*HI sites of pJH101 (see Fig. 2)
pHT1031	20.4	Ap Cm	+	+	pJH101 + pHT 1030; cloning at the *Bam*HI site
pHT1032	20.4	Tc Cm			pJH101 + pHT1030; cloning at the *Pst*I site
pHT1035	6.3	Cm	+	+	The 2.8-kb *Bal*I fragment containing the replication region of pHT1030 is cloned in pJH101 between the *Hin*cII and *Bal*I sites (see Fig. 2).

[a] The phenotypes are those expressed in *E. coli*. In *B. subtilis*, only the resistance to chloramphenicol is expressed.

[b] Replication of the recombinant plasmids in *E. coli* (*E. c*) and *B. subtilis* (*B. s*) is marked +; absence of replication is indicated by the sign −.

Selection of *B. subtilis* transformants was performed on Luria–Bertani (LB) medium plates containing chloramphenicol (10 μg/ml). Selection of *E. coli* transformants was performed on LB plates containing ampicillin (100 μg/ml), tetracycline (5 μg/ml) or chloramphenicol (5 μg/ml) as appropriate.

3.4. Assay of segregational plasmid stability in B. subtilis

Recombination clones grown in LB plates containing chloramphenicol (LB Cm) were used to inoculate 20 ml of LB medium which was incubated overnight without selective pressure at a given temperature (30, 37, 45 or 51°C). The cultures were diluted 1 in 10^3 in 20 ml of fresh LB medium; after about 8 h of incubation with shaking at the desired temperature, the same operation was repeated once in order to reach 30 generations. From the final culture, different dilutions were plated on LB medium to determine the total number of viable cells and on LB Cm medium to determine the number of plasmid-containing bacteria. The LB and LB Cm plates were incubated at the temperature corresponding to the culture. A second estimation was obtained by transferring at least 50 individual colonies from LB plates to LB Cm plates when the proportion of plasmid-containing cells was greater than 1×10^{-2}. The number of Cm^R colonies divided by the total number of colonies represents the frequency of segregational stability of a given plasmid.

4. RESULTS

4.1. Identification and cloning of two native plasmids of B. thuringiensis*: pHT1000 and pHT1030*

The extrachromosomal DNA of strain *thuringiensis* LM2 is constituted of seven plasmids with sizes of 4, 5.6, 8.6, 12, 15, 72 and 80 kb, as determined by agarose gel electrophoresis. Molecular hybridization experiments performed with ^{32}P-labelled specific probes corresponding to Tn*4430* and IS*231* elements showed that the plasmids of 8.6, 15 and 72 kb contain a region that hybridizes with the transposon Tn*4430* and that the plasmids of 15 and 72 kb carry at least one region which

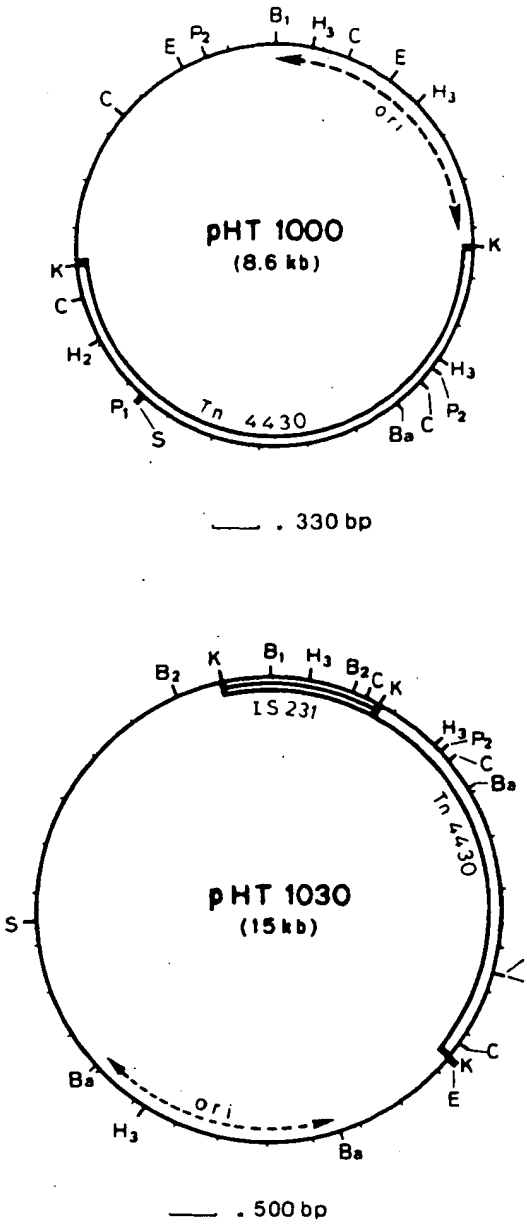

Fig. 1. Restriction maps of two plasmids of *B. thuringiensis*: pHT1000 and pHT1030.

Abbreviations used for the restriction endonuclease sites are as follows: Ba = *Bal*I; B1 = *Bam*HI; B2 = *Bgl*II; C = *Cla*I; E = *Eco*RI; H2 = *Hin*cII; H3 = *Hin*dIII; K = *Knp*I; P1 = *Pst*I; P2 = *Pvu*II; S = *Sst*I. The *Hin*cII sites of pHT1030 are not shown. Ori indicates the replication regions of pHT1000 and pHT1030 determined as described in the text and in Fig. 2.

hybridizes with the IS*231* element (results not shown).

The 8.6- and 15-kb plasmids, respectively designated as pHT1000 and pHT1030, were purified through agarose gel electrophoresis by electroelution and tested for digestion by different restriction endonucleases. Results indicated that each plasmid possessed a unique site for the *Bam*HI and *Pst*I enzymes. The plasmids pHT1000 and 1030 were ligated with the vector pJH101 (see Table 1) after digestion with *Bam*HI or *Pst*I endonuclease. The four ligation mixtures were used to transform *E. coli* strain HB101 and transformants were selected on medium containing ampicillin or tetracycline according to the cloning sites. The recombinant plasmids obtained were designated as pHT1001, 1002, 1031 and 1032 (see Table 1 for description).

4.2. Physical maps of the pHT1000 and pHT1030 plasmids

The restriction maps of pHT1000 and pHT1030 presented in Fig. 1 were deduced from those of the four recombinant plasmids cloned in HB101. The size of these hybrid plasmids is as expected and the patterns of restriction fragments are consistent with their respective cloning sites in pJH101. Thus, the physical maps shown in Fig. 1 are believed to reflect the native plasmids of *B. thuringiensis*.

The comparison of these restriction maps with those of Tn*4430* and IS*231*a [7,9], clearly indicates the position of the putative transposable elements on pHT1000 and pHT1030 (Fig. 1). It appears that the ends of Tn*4430* and IS*231*a are delimited by two *Kpn*I sites as previously reported [7,9]. In the recombinant plasmids pHT1002 and 1032, the vector pJH101 is inserted in Tn*4430* whereas in pHT1031 it is inserted inside the IS*231*a element.

4.3. Localization of the replication regions of the pHT1000 and pHT1030 plasmids

The recombinant plasmids pHT1001, 1002, 1031 and 1032 were used to transform *B. subtilis* strain 168. In each case, transformants resistant to chloramphenicol were obtained. In order to locate

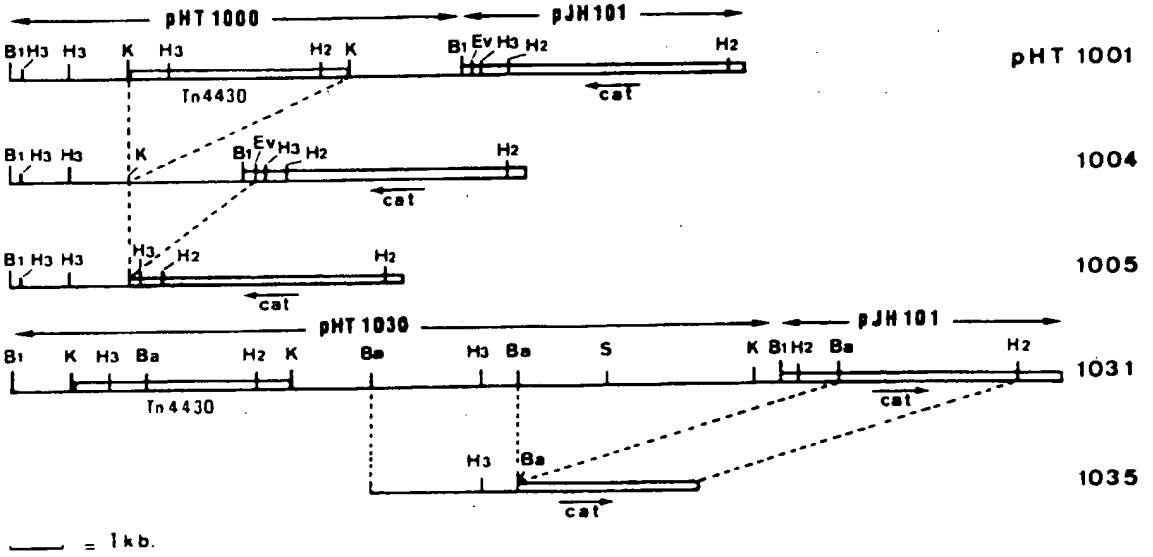

Fig. 2. Construction of the pHT1000- and pHT1030-derived plasmids: cloning of the replication regions functional in *B. subtilis*.

Abbreviations are the same as in Fig. 1; in addition: Ev = *Eco*RV, cat = chloramphenicol acetyl transferase gene of pC194.

Construction of the plasmids is as described in the text and in Table 1. The dashed lines indicate the position of the DNA segments deleted in pHT1004 and pHT1005 or the DNA regions ligated together in pHT1035. For the construction of pHT1005, the digestion at the *Kpn*I site was carried out with *Asp*718 which allows filling in with DNA polymerase I large fragment, generating a blunt end which may be ligated to ends produced by *Eco*RV.

the DNA regions required for the replication of these plasmids in *B. subtilis*, subcloning and deletion experiments were carried out from pHT1001 and pHT1031. Figure 2 shows the construction of the derivatives pHT1004, 1005 and 1035 which replicate in *B. subtilis* and confer resistance to chloramphenicol. Thus, it appears that the replication region of the pHT1000-derived plasmids is containing in a 2.1-kb *Bam*HI-*Kpn*I DNA fragment and that the region which ensures the replication of the plasmids belonging to the pHT1030 series is contained in a 2.8-kb *Bal*I fragment.

The analysis of the plasmid content of the *B. subtilis* transformant clones indicates that the different recombinant plasmids have not undergone any apparent structural rearrangement as compared to the same plasmids extracted from *E. coli* (results not shown).

4.4. Segregational stability and thermosensitivity of the pHT1000 and pHT1030 derived plasmids in B. subtilis

The segregational stability of the various recombination plasmids at 30, 37, 45 and 51°C is shown in Table 2. In each experiment, we used the bifunctional plasmid pHV33 (see Table 1 for origin and construction) as a control. This plasmid is not stably maintained in *B. subtilis* (about 80% loss of Cm^R after 30 generations of growth without selective pressure), but no significant temperature-dependent variation of stability is observed. However, the stability of the pHT plasmids is inversely proportional to the temperature, and neither plasmid is maintained at 51°C.

Table 2

Segregational stability and thermosensitivity of the pHT1000- and pHT1030-derived plasmids in *B. subtilis*

Plasmids	Frequency of segregational stability [a]			
	30°C	37°C	45°C	51°C
pHT1001	5×10^{-1}	3×10^{-2}	10^{-4}	$<4\times10^{-8}$
pHT1002	3×10^{-1}	4×10^{-2}	2×10^{-4}	ND
pHT1004	5×10^{-1}	5×10^{-2}	5×10^{-4}	ND
pHT1005	3×10^{-1}	6×10^{-2}	2×10^{-6}	$<4\times10^{-8}$
pHT1031	9×10^{-1}	5×10^{-1}	5×10^{-2}	$<4\times10^{-8}$
pHT1032	1	5×10^{-1}	9×10^{-2}	ND
pHT1035	9×10^{-1}	3×10^{-1}	10^{-1}	$<4\times10^{-8}$
pHV33	2×10^{-1}	10^{-1}	2×10^{-1}	2×10^{-1}

[a] The frequencies of segregational stability are estimated as described in Section 3 after 30 generations without selective pressure. For frequencies $\geq10^{-2}$ the results represent averages of the two kinds of estimation described.

ND = not determined.

At 30°C, the segregational stability of the pHT1000-derived plasmids is in the same order of magnitude as pHV33, whereas the plasmids derived from pHT1030 are stably maintained, since about 90% of the clones recovered after 30 generations are resistant to chloramphenicol. It should be noted that no significant differences of stability were observed among the various plasmids originating from the same replicon pHT1000 or pHT1030, except for the derivative pHT1005 whose stability decreases much more rapidly as early as 45°C.

The possible effect of the plasmids on the growth rate of host cells was examined. *B. subtilis* strain 168 containing either no plasmid, pHT1001, pHT1031 or pHV33 was grown at 37°C in LB medium and the growth rate measured. No detectable difference in the growth rate of these strains was observed and this is presumably the case at other temperatures. It is therefore unlikely that the values given for the segregational stability of the plasmids are artefacts generated from significant growth rate variations.

5. DISCUSSION

The replication functions of two resident plasmids of *B. thuringiensis*, pHT1000 and pHT1030, were used to construct derivatives able to replicate in *B. subtilis*. The presence of the transposon Tn*4430* and their thermosensitive replication confers an additional interest to these plasmids in view of genetic studies in gram-positive bacteria.

Moreover, the present investigations suggest two hypotheses relating to the segregational stability of plasmids in *B. subtilis*. First, the site-specific recombination system of Tn*4430* (Mahillon, and Lereclus, D., unpublished data), if expressed in *B. subtilis*, does not interfere with the stability of

plasmids, which is not the case in *E. coli* where it has been shown that site-specific resolution of multimers increases the maintenance of bacterial replicons [15,16]. Indeed, significant differences of stability are not observed with the derivatives pHT1004, 1005 and 1035 in which Tn*4430* is missing or with pHT1002 and 1032 in which the resolution gene is disrupted by insertion of pJH101 in the *Pst*I site (unpublished data). Secondly, it appears that the stability of the various recombination plasmids examined is not dependent on their size, which contrasts with the negative correlation between the size of pUB110-derived plasmids and their segregational stability in *B. subtilis* [17].

These plasmids may be useful tools for the study of plasmid replication and the transposition of Tn*4430* in *B. thuringiensis* once a reproducible transformation system is available for this organism.

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Construction and Properties of an Integrable Plasmid for *Bacillus subtilis*

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A plasmid useful for locating the chromosomal site of cloned DNA fragments that lack intrinsic genetic activity, for insertional mutagenesis, for single-copy complementation, and for dominance studies was constructed. Some plasmids containing *Bacillus subtilis* DNA were only active in transformation when the tetracycline resistance determinant of the plasmid was inactivated. The results suggest that production of the tetracycline gene product is lethal to *B. subtilis*.

Plasmids containing a region of homology with the *Bacillus subtilis* chromosome integrate at high frequency into the chromosome by Campbell-type recombination (2, 4, 5). Such integrated plasmids have proven useful for studying the chromosomal location of cloned fragments by classical methods of transformation and transduction. If the plasmid contains an expressable gene such as for chloramphenicol resistance, the integrated plasmid can be located in genetic crosses by this phenotype (4). This property adds a powerful tool to systems of genetic analysis of *B. subtilis*, as cloned DNA fragments with no inherent genetic phenotypes can be analyzed with relative ease. To take full advantage of this system, we constructed a plasmid vector with a variety of restriction sites, with an expressable chloramphenicol resistance gene, and without any ability to replicate freely in *B. subtilis*.

To obtain a gene capable of expression in both *Escherichia coli* and *B. subtilis* that would allow direct selection in both organisms, the chloramphenicol resistance gene from *Staphyloccus aureus* plasmid pC194 was inserted into the unique *Pvu*II site of *E. coli* plasmid pBR322. The chloramphenicol acetyl transferase gene of pC194 is known to be entirely contained on an approximately 1-kilobase restriction fragment that can be generated by double digestion with restriction endonucleases *Mbo*I and *Hpa*II (6). These enzymes were used to digest pC194, and the chloramphenicol acetyl transferase-containing restriction fragment was further digested with S1 exonuclease to remove the single-stranded end generated by the *Mbo*I enzyme. The S1-treated, chloramphenicol acetyl transferase-containing fragment was ligated with T_4 ligase to pBR322 that had been previously digested with restriction endonuclease *Pvu*II. The ligation mixture was used to transform *E. coli* 294, with simultaneous selection for ampicillin resistance (Ap^r), tetracycline resistance (Tet^r), and chloramphenicol resistance (Cm^r). Plasmids from several colonies that arose on these plates were analyzed for size by electrophoresis and for their ability to transform all three antibiotic resistances. The restriction map of one of these, JH101, is shown in Fig. 1. The orientation of the chloramphenicol acetyl transferase fragment was determined from the location of internal *Hae*III restriction sites.

pJH101 does not contain an origin of replication for *B. subtilis* and cannot replicate in *B. subtilis*. Thus, competent cells do not yield Cm^r transformants after exposure to pJH101. If any region of homology with the *B. subtilis* chromosome is inserted within pJH101, however, the recombinant plasmid gains the ability to transform competent cells at high frequency. Table 1 shows the results of transformation experiments using plasmid pDL44, which consists of pJH101 containing the *Eco*RI-*Hin*dIII fragment which carries the *pheA* locus (Fig. 2). pDL44 was used as a donor to transform JH247, with independent selection for Phe^+ and Cm^r. Phe^+ transformants can arise either by insertion of the plasmid intact within the chromosome by reciprocal recombination or by normal transformation using a double-crossover event in which the nonhomologous plasmid is excluded. Examination of the Phe^+ transformants in this cross revealed that 20% of them (Phe^+ Cm^r) arose by the former mechanism and 80% (Phe^+ Cm^s) arose by the latter. Direct selection for the reciprocal event, i.e., Cm^r, always yielded 2- to 10-fold fewer

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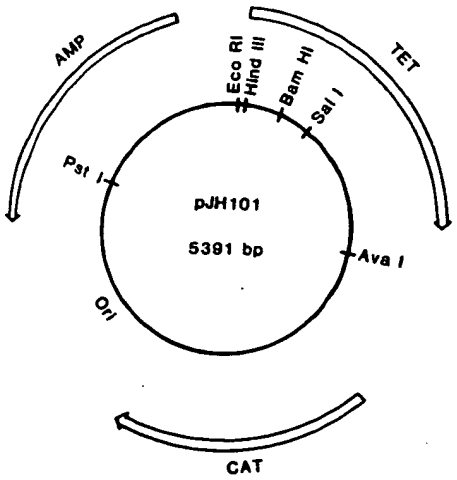

FIG. 1. Restriction map of pJH101.

recombinants. In this case, all 240 Cm^r recombinants examined were Phe^+, but this result depends upon the fragment cloned and the placement of the gene of interest within the fragment.

In the cases examined, the reciprocal recombination event that inserts pJH101 (3) or other plasmids of this type (4) occurred by Campbell-type recombination. This resulted in a duplicated region on either side of the plasmid which should allow crossing over, excision, and loss of the integrated plasmid at a detectable frequency. In several experiments to measure this frequency, it was found that the integrated plasmids were quite stable in that configuration. Strains bearing pDL44 did not lose this plasmid after 5 to 10 generations in the absence of selection for Cm^r. Similar strains growing for more than 50 generations in the absence of selection for Cm^r lost the plasmid at a frequency of between 1 and 5%. Several factors may enter into this frequency, such as the length of the duplicated region and the genetic background of the strain.

pJH101 has been successfully used to clone and map by insertion several areas of the *B. subtilis* chromosome (3; unpublished data). A curious result was obtained when plasmids

TABLE 1. Transformation of the *pheA* locus by a pJH101 derivative.

Donor	Recipient	Transformant	No./ml	Recombinant classes (no.)		
				Phe^+ Cm^s	Phe^+ Cm^r	Phe^- Cm^r
pDL44[a]	JH247[b]	Phe^+	2.5×10^4	201	44	
		Cm^r	1.9×10^4		45	195
W168	JH247	Phe^+	1.0×10^5			
		Cm^r	0			

[a] DNA used at μg/ml, as in the method of Anagnostopolus and Spizizen (1).
[b] JH247 (*trpC2 phe-1*).

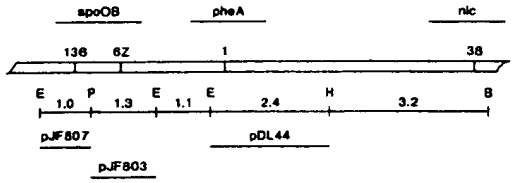

FIG. 2. Physical map of the *spo0B-nic* region of the chromosome and location of the DNA fragments cloned in pJH101. Numbers indicate distances between restriction endonuclease sites in kilobase pairs. Restriction endonucleases: E, *Eco*RI; P, *Pst*I; H, *Hin*dIII; B, *Bam*HI.

pJF803 and pJF807 were prepared by cloning *Eco*RI-*Pst*I fragments of the *Eco*RI fragment containing *spo0B* (Fig. 2). Neither pJF803 or pJF807 gave Cm^r transformants when exposed to competent cells, yet both plasmids were capable of transforming *spo0B* alleles to prototrophy (Table 2). We considered two possible causes for this phenomenon. Perhaps the integration of the plasmid within this region disrupted the regulation of some gene(s) within or adjacent to the region that was required for growth. Alternatively, the integration of these plasmids might have activated some normally cryptic plasmid gene whose presence was lethal to the cell. One likely candidate for such a cryptic gene is the tetracycline resistance determinant immediately adjacent to the cloned fragment. To determine whether the tetracycline resistance determinant was involved in this phenomenon, the determinant was inactivated by *Bam*HI endonuclease digestion followed by S1 nuclease digestion and religation. The ligation mixture was transformed into competent *E. coli* cells, with selection for ampicillin resistance, and tetracycline-sensitive transformants were identified. Two of these, pJF908 and pJF910 (derived from pJF803 and pJF807, respectively), were studied further.

pJF908 and pJF910 were fully capable of transforming competent cells to chloramphenicol resistance, unlike their parental plasmids (Table 2). Thus, the inability of certain recombinant plasmids to transform *B. subtilis* is related to some lethal consequence of activation of the tetracycline resistance determinant. Therefore, it seems likely that the 2.3-kilobase *Eco*RI-*Eco*RI fragment containing the *spo0B* locus has promoters, at both ends, facing away from the middle of the fragment. The promoter for the tetracycline resistance determinant of pBR322 is thought to be active in *B. subtilis* and give rise to tetracycline resistance (7). Our results can be rationalized if overproduction of a tetracycline resistance gene product is lethal.

A collection of plasmids containing random fragments of the *B. subtilis* chromosome was generated. Chromosomal DNA was digested to completion with restriction endonuclease

TABLE 2. Transformation of *spo0B* alleles with plasmids containing a fragment of the *spo0B* locus

Donor	Recipient	Transformants/ml		Recipient	Transformants/ml	
		Cm^r	Spo^+		Cm^r	Spo^+
pJF807[a]	JH648[b]	0	8.0×10^2	6Z[c]	0	0
pJF803	JH648	0	0	6Z	0	6.3×10^2
pJF910	JH648	4.2×10^3	0.5×10^2	6Z	1.3×10^2	0
pJF908	JH648	5.3×10^3	0	6Z	6.0×10^2	0.7×10^2
pJF840	JH648	3.2×10^3	0	6Z	1.0×10^3	0
W168	JH648	0	9.6×10^2	6Z	0	5.5×10^2

[a] All DNA were used at 1 μg/ml, as in the method of Anagnostopolus and Spizizen (1).
[b] JH648 *trpC2 phe-1 spo0B136*.
[c] 6Z *trpC2 spo0B6Z*.

*Sau*3A. Plasmid pJH101 was digested with restriction endonuclease *Bam*HI and mixed with a threefold excess of the *Sau*3A-digested chromosomal DNA. The mixture was subjected to ligation with T_4 ligase. The ligation mixture was used to transform *E. coli* for ampicillin resistance (Amp^r). Tetracycline-sensitive transformants comprised 27% of the Amp^r transformants. About 1,500 of the Tet^s clones were pooled, and a plasmid preparation was obtained from this pool.

The pooled plasmid preparation was used as a donor to transform strain 168 for Cm^r. The transformants obtained were scored for the Spo^+/Spo^- phenotype and replica plated to minimal medium containing tryptophan for detection of auxotrophic transformants. One hundred thirty-three Spo^- colonies were found among 3,008 Cm^r transformants. The same transformants yielded 10 auxotrophic mutants. The exact requirements of these mutants were not determined, although several were noted to respond to amino acid supplementation. This compares favorably with the results of similar experiments, using plasmid pHV32, by B. Niaudet and S. D. Ehrlich (personal communication). These investigators also showed that the plasmid can be recovered from the mutant with the inserted region by cleavage of the chromosomal DNA of the mutant with restriction enzymes that do not cleave the plasmid, followed by religation and transformation in *E. coli*. Identical results have been obtained with integrated plasmid pJH101 (our unpublished data; E. Ferrari, unpublished data). It should be noted that mutants are generated by this method only if the cloned fragment is entirely contained within a transcriptional unit.

Since the chromosomally inserted plasmid can be easily recovered by transformation into *E. coli*, it is possible to directly clone any selectable locus by transformation with pools of plasmids such as those prepared as described above for mutagenesis. Selection for prototrophy should yield some transformants with the plasmid integrated (i.e., Cm^r). The locus of interest can be recovered by the *E. coli* transformation technique. Although at first glance this may appear to be an oblique approach to the problem of cloning a locus, it has the advantage that a genetically active DNA fragment for the locus in question will yield transformants and, therefore, some of the locus in cloned form irregardless of whether the locus is intact on the DNA fragment.

The inability of pJH101 to replicate in *B. subtilis* confers an additional use for this plasmid. Complementation and dominance studies of cloned genes can be undertaken without the complication of a large excess of one of the complementing alleles due to replication of the plasmid containing the cloned gene.

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